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Article

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Linkage of β_1 -adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca^{2+} /calmodulin kinase II

See the related Commentary beginning on page 597.

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β_1 -adrenergic receptor (β_1 AR) stimulation activates the classic cAMP/protein kinase A (PKA) pathway to regulate vital cellular processes from the change of gene expression to the control of metabolism, muscle contraction, and cell apoptosis. Here we show that sustained β_1 AR stimulation promotes cardiac myocyte apoptosis by activation of Ca^{2+} /calmodulin kinase II (CaMKII), independently of PKA signaling. β_1 AR-induced apoptosis is resistant to inhibition of PKA by a specific peptide inhibitor, PKI14-22, or an inactive cAMP analogue, Rp-8-CPT-cAMPS. In contrast, the β_1 AR proapoptotic effect is associated with non-PKA-dependent increases in intracellular Ca^{2+} and CaMKII activity. Blocking the L-type Ca^{2+} channel, buffering intracellular Ca^{2+} , or inhibiting CaMKII activity fully protects cardiac myocytes against β_1 AR-induced apoptosis, and overexpressing a cardiac CaMKII isoform, CaMKII- δC , markedly exaggerates the β_1 AR apoptotic effect. These findings indicate that CaMKII constitutes a novel PKA-independent linkage of β_1 AR stimulation to cardiomyocyte apoptosis that has been implicated in the overall process of chronic heart failure.

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Introduction

Stimulation of β -adrenergic receptor (β AR), a prototypical G protein-coupled receptor, is broadly involved in metabolic regulation, growth control, muscle contraction, cell survival, and cell death. In the heart, β AR stimulation by catecholamines serves as the most powerful regulatory mechanism to enhance myocardial performance in response to stress or exercise by activating the classic stimulatory pathway comprising the G protein G_s , adenylyl cyclase, cAMP, and protein kinase A (PKA) (1, 2). However, sustained activation of β_1 AR, the predominant β AR subtype expressed in the heart, also induces cardiac myocyte apoptosis (3–6).

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Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: β -adrenergic receptor (β AR); protein kinase A (PKA); Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII); isoproterenol (ISO); pertussis toxin (PTX); Rp-8-CPT-cAMPS (Rp-cAMP); protein kinase A inhibitor 14-22 (PKI); autocalmitide-2-related inhibitory peptide (AIP); sarcoplasmic reticulum (SR); hemagglutinin (HA); phospholamban (PLB).

Apoptotic heart cell death has been implicated in the overall process of myocardial remodeling and the transition from cardiac hypertrophy to chronic heart failure (7–10), an illness afflicting more than five million Americans, with a 5-year mortality greater than 80% (11). However, the mechanism underlying the β_1 AR apoptotic effect remains poorly understood.

Previous studies suggested that β_1 AR-induced cardiac myocyte apoptosis was mediated by the cAMP/PKA pathway (12, 13), the only known intracellular mechanism underlying β_1 AR-elicited cellular responses (1, 2). However, transgenic overexpression of type V (14) or type VI (15) adenylyl cyclase in mouse hearts does not cause cell death, although it markedly augments basal PKA activity (14) and cardiac contractility (14, 15). More ironically, in cultured cardiac myocytes or in vivo, selective β_2 AR subtype stimulation elicits a profound cardiac protective effect (4, 5, 16), in spite of overtly enhanced cAMP formation (16–18).

The goal of the present study is to determine the mechanism of β_1 AR-induced apoptosis. In addition to pharmacological approaches used in WT mouse cardiac myocytes, we created genetically “pure” β_1 AR experimental settings using adult cardiac myocytes from β_2 AR KO mice (19) or by adenovirus-mediated gene transfer (20) of the mouse β_1 AR in myocytes from $\beta_1\beta_2$ double knockout (DKO) mice (21). These approaches enabled us to avoid the complicated interactions between the

coexisting β_1 AR and β_2 AR subtypes that exert opposing effects on cardiac cell survival and cell death (4, 5, 16). In the present study, we demonstrate that β_1 AR-induced cardiac myocyte apoptosis is independent of cAMP and PKA signaling, but requires a novel signaling pathway involving Ca^{2+} and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII).

Methods

Materials. Isoproterenol (ISO); norepinephrine; prazosin; propranolol; cyclosporin A; FK506; ICI 118,551; H-89; pertussis toxin (PTX); nifedipine; and thapsigargin were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Rp-8-CPT-cAMPS (Rp-cAMP) was purchased from BIOLOG Life Science Institute (La Jolla, California, USA). PKI14-22 (PKI), autocalmitide-2-related inhibitory peptide (AIP), KN93, and KN92 were purchased from Calbiochem-Novabiochem Corp. (La Jolla, California, USA).

Cardiac myocyte culture and adenoviral infection. Single cardiac myocytes were enzymatically isolated from the hearts of 2- to 3-month-old WT, β_2 AR KO, or $\beta_1\beta_2$ DKO male mice, and then cultured. $\beta_1\beta_2$ DKO and β_2 AR KO cells were infected with target gene-carrying adenoviral vectors as described previously (20). Briefly, myocytes were plated at 0.5×10^4 to 1×10^4 cells per cm^2 with MEM containing 1.2 mM Ca^{2+} and 1% penicillin-streptomycin in culture dishes precoated with 10 $\mu\text{g}/\text{ml}$ mouse laminin. Adenovirus-mediated gene transfer was implemented by adding adenoviral vectors carrying either the mouse β_1 AR gene (Adv- β_1 AR), the marker gene β -gal (Adv- β -gal), or the β ARK-ct gene (Adv- β ARK-ct, kindly provided by R.J. Lefkowitz and W.J. Koch, Duke University, Durham, North Carolina, USA) to $\beta_1\beta_2$ DKO cells. Alternatively, hemagglutinin-tagged (HA-tagged) CaMKII- δ B vector (Adv-CaMKII- δ B) or HA-tagged CaMKII- δ C vector (Adv-CaMKII- δ C) was added to β_2 AR KO cells. All transfections were at an MOI of 100. At the MOI used, almost 100% of myocytes were positively infected, as evidenced by β -gal staining (20) or GFP fluorescent signal (Adv-GFP infection, our unpublished data).

After adenoviral infection for 24 hours, culture medium was added with designated reagents, including PKA inhibitors (PKI or Rp-cAMP), CaMKII inhibitors (AIP, KN93, or KN93's inactive analogue, KN92) 1 hour prior to β_1 AR stimulation by ISO. In addition, calcineurin inhibitors (cyclosporin A or FK506), β AR antagonists (propranolol or ICI 118,551), or an L-type Ca^{2+} channel antagonist (nifedipine), or a sarcoplasmic reticulum (SR) Ca^{2+} pump inhibitor (thapsigargin) were added 1 hour prior to ISO treatment (1 μM) in some subsets of experiments. All apoptosis assays were performed after ISO treatment for 24 hours unless otherwise indicated. All dishes were supplemented with ascorbic acid (100 μM ; Sigma-Aldrich) to prevent ISO oxidation.

TUNEL and Hoechst staining. Nuclear fragmentation was determined in fixed cells (70% alcohol and 30% acetone) either by incubating in 10 μM Hoechst 33342 or

by TUNEL staining with apoptosis detection kits (R&D Systems Inc., Minneapolis, Minnesota, USA), as previously described (5). The percentage of TUNEL- or Hoechst-positive cells was determined by counting 500–800 cardiac myocytes in 20 randomly chosen fields in each culture dish (with cells pooled from two to three hearts). (In Figure 1, c and d, Figure 2, Figure 4c, Figure 5b, and Figure 7c, all n values refer to the number of independent experiments, and each data point shows the result from 5,000–6,000 cells, $n = 4$ –8.) As shown previously (5), the percentage of TUNEL-positive cells is lower than that of Hoechst-positive cells because some apoptotic myocytes are detached and washed away during the TUNEL staining assay.

DNA laddering and cell death ELISA. For DNA laddering, 10 μg of DNA was loaded in each lane. The DNA was size-fractionated on a 1.5% agarose gel in Tris-acetate-EDTA buffer and then stained with ethidium bromide (Life Technologies Inc., Carlsbad, California, USA).

For cell death ELISA, myocytes in each group were harvested by trypsinization and then combined with the cells pelleted from media. The cell pellets were washed with PBS and then split into two aliquots: one used for assay of protein abundance to normalize among samples, and the other for extraction of cytoplasmic DNA. DNA was assayed using a commercially available kit to measure nucleosomal DNA (Cell Death Detection ELISA Plus; Roche Molecular Biochemicals, Indianapolis, Indiana, USA). Specifically, 0.20 ml of lysis buffer was used for each aliquot of approximately 100,000 cells. Cells were lysed by gently dispersing the pellet using a pipette tip that was cut back to prevent shearing of cells and release of nuclear DNA. The extraction was performed at room temperature and took 30 minutes. The extract was then centrifuged at low speed and assayed according to the manufacturer's instructions. The control absorbance, measured in myocytes infected by Adv- β_1 AR, was considered as 100% or one unit. Fold increase was obtained by dividing the measured absorbance of an experimental group by the absorbance of the control. All measurements were normalized to the amount of total cellular protein. All measurements were made in triplicate and analysis was performed on at least four independent experiments.

Measurement of intracellular Ca^{2+} . Cultured myocytes in the absence of electrical pacing were loaded with a fluorescent Ca^{2+} probe, indo-1-acetoxymethyl ester (Molecular Probes Inc., Eugene, Oregon, USA), and were excited at 350 nm. The ratio (R) of fluorescence emission at 410 nm to that at 490 nm was used as an index of intracellular Ca^{2+} , as described previously (22). The intracellular free Ca^{2+} concentration was calculated according to the equation $[\text{Ca}^{2+}]_i = K_d \beta (R - R_{\min}) / (R_{\max} - R)$, where R_{\min} and R_{\max} are the ratio of fluorescence signal at 490 nm at zero and saturating $[\text{Ca}^{2+}]_i$, respectively; β is a constant, and K_d is the dissociation constant of the indicator. In addition, spatial properties of the caffeine-releasable SR Ca^{2+} store were examined using an LSM410 confocal microscope (Carl Zeiss Jena GmbH, Jena, Germany) with

UV laser excitation (351 nm) and dual wavelength ratio-metric (410/490 nm) imaging. In another subset of experiments, intracellular Ca^{2+} transients were measured in cultured myocytes electrically paced at 0.5 Hz.

Phospholamban Ser¹⁶ phosphorylation, CaMKII autophosphorylation, and CaMKII activity assay. PKA-dependent phosphorylation of phospholamban (PLB) at Ser¹⁶ was detected by Western blot using a site-specific antibody (Badrilla, West Yorkville, United Kingdom). Total CaMKII protein abundance was assayed by Western blot using an antibody reacting with total CaMKII (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), whereas autophosphorylated CaMKII was determined with a monoclonal antibody reacting with phosphorylated CaMKII (Affinity BioReagents Inc., Golden, Colorado, USA) (23). CaMKII activity was evaluated with CaMKII assay kits (Upstate Biotechnology Inc., Lake Placid, New York, USA) and a peptide substrate (KKALRRQETVDAL) of the kinase.

Western blot analysis and confocal immunocytochemical imaging of HA-tagged CaMKII- δ B or CaMKII- δ C. Adenovirus-directed expression of HA-tagged CaMKII- δ B or CaMKII- δ C in β_2 AR KO cardiomyocytes was examined by Western blot with a monoclonal antibody reacting with HA (Covance Inc., Princeton, New Jersey, USA). To determine subcellular localization of CaMKII- δ C, cells were incubated overnight at 4°C with the HA monoclonal antibody, followed by an incubation for 4 hours with a Texas Red-conjugated anti-mouse antibody (Vector Laboratories Inc., Burlingame, California,

USA). Immunostaining was then visualized with a scanning confocal microscope (LSM510; Carl Zeiss Jena GmbH), as described previously (23).

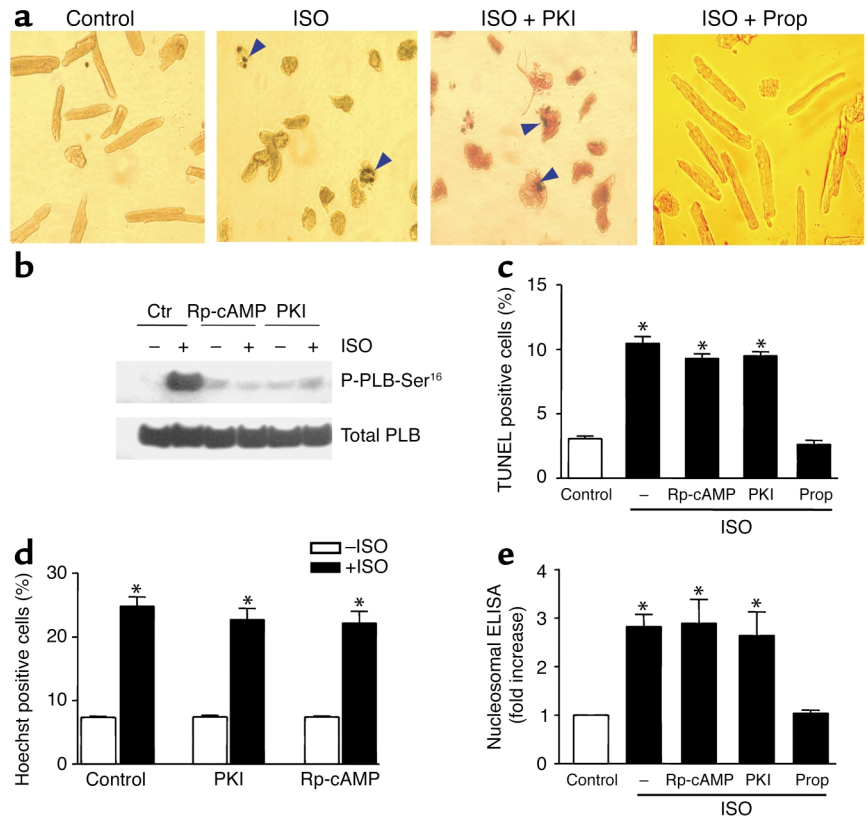
Statistical analysis. Data were expressed as mean \pm SE. Statistical comparisons used one-way ANOVA followed by the Bonferroni procedure for multiple-group comparisons. $P < 0.05$ was considered statistically significant.

Results

Sustained β_1 AR stimulation delivers a potent apoptotic signal in cardiac myocytes. In $\beta_1\beta_2$ DKO cells infected with an adenoviral vector encoding β_1 AR (Adv- β_1 AR) at an MOI of 100 for 24 hours, the β_1 AR density was 550 ± 46 fmol/mg protein ($n = 4$), which was approximately 16-fold greater than the receptor density in WT cells (33.5 ± 1.5 fmol/mg protein, $n = 3$). Stimulation of the expressed β_1 ARs by ISO (1 μ M) for 24 hours caused cardiomyocyte death by increased apoptosis (Figure 1a). β_1 AR stimulation led to a threefold augmentation in cells positive for either TUNEL (Figure 1, a and c) or Hoechst staining (Figure 1d). Apoptotic nuclei appeared blue by TUNEL staining, with a varying degree of chromatin condensation and fragmentation, as indicated by arrows (Figure 1a). Concomitantly, there was a marked increase in DNA fragmentation assayed by cell death ELISA (Figure 1e) and DNA laddering (see Figure 5c) in myocytes subjected to ISO treatment. Propranolol (10 μ M), a β AR antagonist, protected myocytes from ISO-induced apoptosis (Figure 1, a, c, and e, and Figure 5c), indicating that the ISO effect is mediated by

Figure 1

Inhibition of the cAMP/PKA signaling pathway did not protect myocytes from β_1 AR-induced apoptosis. Cardiac myocytes from $\beta_1\beta_2$ DKO mice were infected with either Adv- β_1 AR or Adv- β -gal at an MOI of 100. (a) Typical micrographs of TUNEL staining of myocytes. Treatment with 1 μ M ISO for 24 hours increased the number of apoptotic cells (arrows); the β AR antagonist propranolol (10 μ M), but not the PKA inhibitor PKI (5 μ M), prevented the β_1 AR apoptotic effect. (b) ISO-induced phosphorylation of PLB at Ser¹⁶ (P-PLB-Ser¹⁶) in the absence (Ctr) or presence of Rp-cAMP (100 μ M) or PKI (5 μ M). Similar results were obtained in four other experiments. Pretreatment periods of 1 hour (shown) and 6 hours (not shown) of cells with the PKA inhibitors were equally effective in blocking PKA-dependent PLB phosphorylation in response to ISO treatment (1 μ M for 10 minutes). (c-e) Effects of PKA inhibitors on ISO-induced increase in TUNEL staining (c), Hoechst staining (d), or DNA fragmentation as assayed by cell death ELISA (e). Data are presented as mean \pm SE ($n = 4-8$ independent experiments in 5,000-6,000 cells from 10-20 hearts for each group). * $P < 0.01$ vs. ISO-untreated cells or those pretreated with propranolol. Prop, propranolol.



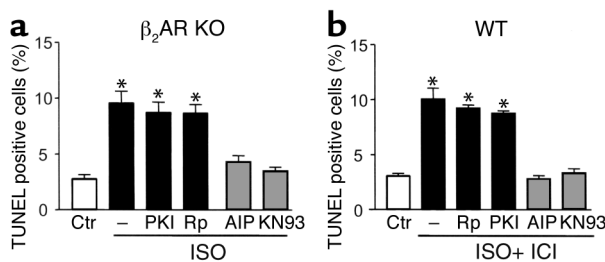


Figure 2

Effects of PKA and CaMKII inhibitors on β_1 AR-mediated increase in TUNEL-positive cells in β_2 AR KO (a) or WT (b) mouse cardiac myocytes. β_1 ARs in β_2 AR KO myocytes were stimulated with 1 μ M ISO, and β_1 ARs in WT cells were stimulated with 1 μ M ISO plus the β_2 AR blocker ICI 118,551 (0.5 μ M). * $P < 0.01$ vs. ISO-untreated cells or those pretreated with AIP (10 μ M) or KN93 (0.5 μ M) ($n = 6$ for each group). Rp, Rp-cAMP.

receptor activation. The earliest significant apoptotic effect occurred after 8 hours of ISO treatment, as evidenced by a 2.2-fold increase in the percentage of TUNEL-positive cells ($n = 3$, $P < 0.05$). Similarly, β_1 AR stimulation by ISO markedly increased apoptotic cell death in β_2 AR KO myocytes (Figure 2a). Furthermore, selective β_1 AR stimulation by ISO (1 μ M) in the presence of a β_2 AR blocker, ICI 118,551 (0.5 μ M), clearly increased WT myocyte apoptosis (Figure 2b). These results validate the relevance of our data in DKO cells expressing β_1 AR using adenoviral gene transfer. Thus, sustained β_1 AR stimulation delivers a potent apoptotic signal, in agreement with previous reports (3–6). Since a similar maximal apoptotic effect occurred in all three experimental systems examined, the receptor density appears not to be a rate-limiting factor in transducing β_1 AR apoptotic signal in cardiac myocytes.

β_1 AR-induced apoptosis is independent of cAMP/PKA signaling. To determine the potential role of cAMP/PKA in β_1 AR-mediated myocyte apoptosis, we first used H-89, a widely used PKA inhibitor, and found that H-89 at a high concentration (20 μ M) protected cardiac myocytes against β_1 AR-induced apoptosis. TUNEL-positive cells were reduced from 11.4% \pm 1.1% to 3.3% \pm 0.8% by H-89 ($n = 4$, $P < 0.01$), consistent with previous reports (12). However, the interpretation of experiments using H-89 is complicated by the recent finding that H-89 is also a potent β AR blocker (24). We therefore inhibited this pathway using a highly specific, membrane-permeable peptide PKA inhibitor, PKI (25), and an inactive cAMP analogue, Rp-cAMP (26). Pretreatment of myocytes with either Rp-cAMP (100 μ M) or PKI (5 μ M) completely abolished PKA-dependent phosphorylation of PLB (Figure 1b), a key cardiac PKA target protein that regulates the SR Ca^{2+} pump activity (27), thus validating the effectiveness of these PKA inhibitors in abrogating cAMP/PKA signaling. Surprisingly, neither PKA inhibitor at the same respective concentrations had any significant effect on β_1 AR-mediated apoptosis (Figure 1, a and c–e, and Figure 5c).

The inability of PKA inhibitors to protect cardiac myocytes against β_1 AR-induced apoptosis was confirmed in the β_2 AR KO model, in which the density and functionality of native β_1 ARs remain unaltered (19), and in WT mouse myocytes subjected to ISO in the presence of β_2 AR blockade. Stimulation of the native β_1 AR in adult β_2 AR KO or WT cardiac myocytes by ISO similarly increased TUNEL-positive cells even in the presence of PKI or Rp-cAMP (Figure 2). Thus, while the cAMP/PKA signaling pathway has been thought to be the sole mechanism responsible for β_1 -adrenergic responses, β_1 AR apoptotic signal transduction essentially bypasses this pathway.

Role of $G_{\beta\gamma}$ or G_i signaling. To identify the molecular mediator for the non-PKA-dependent β_1 AR apoptotic effect, we tested several candidates, including free $G_{\beta\gamma}$ released from heterotrimeric G_s proteins or G proteins other than G_s (e.g., G_i proteins). The possible involvement of $G_{\beta\gamma}$ or G_i signaling in β_1 AR-mediated apoptosis was examined by adenoviral gene transfer of the C-terminal domain of β AR kinase (β ARK-ct) to inhibit $G_{\beta\gamma}$ signaling (28) and by pretreatment of cells with PTX to disrupt G_i signaling (29). Neither β ARK-ct nor PTX significantly affected β_1 AR-mediated apoptotic DNA fragmentation (Figure 3). In contrast, both interventions effectively blocked the $G_{\beta\gamma}$ - and G_i -mediated β_2 AR antiapoptotic effect under similar experimental conditions (5) and prevented $G_{\beta\gamma}$ -mediated activation of PI3K (30). These results rule out the possibility that $G_{\beta\gamma}$ or G_i signaling is responsible for β_1 AR-mediated cardiomyocyte apoptosis.

Essential role of Ca^{2+} entry and intracellular Ca^{2+} in β_1 AR apoptotic signaling. It has been shown that altered Ca^{2+} signaling promotes apoptosis in a variety of cell types (31). We next explored the possibility that Ca^{2+} , instead of cAMP, acts as the second messenger to transmit the β_1 AR apoptotic signal in cardiac myocytes. In Adv- β_1 AR-infected DKO myocytes, ISO treatment for 3–6 hours (just prior to manifestation of β_1 AR-induced apoptosis) significantly elevated intracellular free Ca^{2+}

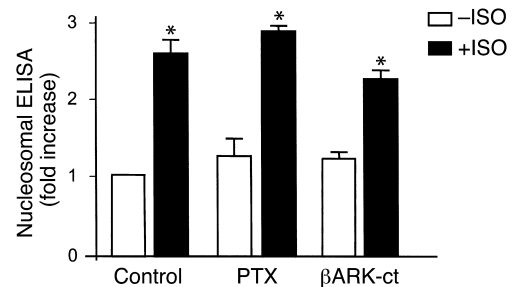


Figure 3

$G_{\beta\gamma}$ or G_i signaling is not involved in β_1 AR-induced cardiomyocyte apoptosis. Neither inhibition of $G_{\beta\gamma}$ signaling by adenoviral expression of β ARK-ct nor disruption of G_i signaling by pretreatment of cells with PTX (1 μ g/ml for 3 hours) altered ISO-induced (1 μ M) DNA fragmentation assayed by cell death ELISA in $\beta_1\beta_2$ DKO cells infected by Adv- β_1 AR. * $P < 0.01$ vs. ISO-untreated myocytes. $n = 6$ –7 independent experiments for each group.

as measured with the Ca^{2+} -sensitive fluorescent probe indo-1. As shown in Figure 4a, sustained $\beta_1\text{AR}$ stimulation increased basal cytosolic Ca^{2+} from 122.8 ± 5.8 ($n = 32$ cells from six hearts) to 308.7 ± 9.7 nM ($n = 29$ cells from six hearts). This Ca^{2+} response remained largely intact in the presence of PKI ($5 \mu\text{M}$) but was abolished by nifedipine ($1 \mu\text{M}$), an L-type Ca^{2+} channel antagonist, indicating an elevation in intracellular Ca^{2+} mediated by L-type Ca^{2+} currents (I_{Ca}) (Figure 4a). Furthermore, we measured phasic intracellular Ca^{2+} transients in cultured, paced (0.5 Hz) cardiac myocytes in the presence and absence of prolonged $\beta_1\text{AR}$ stimulation by ISO. $\beta_1\text{AR}$ stimulation significantly increased both diastolic and systolic Ca^{2+} , with the diastolic effect most prominent, in paced cardiac myocytes (Figure 4b). Thus, $\beta_1\text{AR}$ stimulation increases cytosolic Ca^{2+} in both contracting and noncontracting cardiac myocytes. In order to determine whether the altered Ca^{2+} homeostasis is causally linked to $\beta_1\text{AR}$ -induced apoptosis, we inhibited Ca^{2+} entry by nifedipine or buffered intracellular Ca^{2+} by incubating cells with EGTA-AM. Both treatments rescued cardiac myocytes from $\beta_1\text{AR}$ -induced apoptosis (Figure 4c).

In addition, we measured SR Ca^{2+} load, as indexed by the amplitude of Ca^{2+} transients generated in response to a bolus administration of caffeine ($20 \mu\text{M}$). We found that sustained $\beta_1\text{AR}$ stimulation significantly elevated the caffeine-releasable SR Ca^{2+} content, by 38% (Figure 4, d and e). Confocal imaging further revealed that the caffeine-induced Ca^{2+} release was spatially uniform (at the optical resolution), regardless of $\beta_1\text{AR}$ stimulation (Figure 4d). Paralyzing SR Ca^{2+} recycling by inhibiting the SR Ca^{2+} pump with thapsigargin also protected myocytes from $\beta_1\text{AR}$ -induced apoptosis (Figure 4c). Together, these results indicate that the enhanced L-type channel Ca^{2+} influx, the subsequent increases in cytosolic Ca^{2+} , and the SR Ca^{2+} overload are all required for $\beta_1\text{AR}$ -induced myocyte apoptosis.

$\beta_1\text{AR}$ -induced apoptosis requires PKA-independent activation of CaMKII. To further delineate the specific pathway transducing Ca^{2+} -mediated $\beta_1\text{AR}$ apoptotic signaling, we examined possible roles of Ca^{2+} -dependent protein phosphatases and kinases, particularly calcineurin and CaMKII. It remains controversial whether activation of calcineurin participates in Ca^{2+} -induced cardiac myocyte apoptosis (32, 33), whereas its apoptotic effect is well established in other cell types (34). The present data show that inhibition of calcineurin by cyclosporin A ($5 \mu\text{M}$) or FK506 ($10 \mu\text{M}$) failed to block $\beta_1\text{AR}$ -induced apoptotic heart cell death, suggesting that calcineurin does not play an essential role in $\beta_1\text{AR}$ apoptotic signaling (Figure 5b).

In sharp contrast, a highly specific membrane-permeable peptide inhibitor of CaMKII, autocamtide-2-related inhibitory peptide (AIP, $10 \mu\text{M}$) (35), fully protected myocytes from $\beta_1\text{AR}$ -induced apoptosis, as evidenced by the typical micrographs (Figure 5a) and the average results of TUNEL staining (Figure 5b). Similar protective effects were observed with another specific CaMKII inhibitor, KN93 ($0.5 \mu\text{M}$), but not its inactive analogue, KN92 ($2 \mu\text{M}$) (Figure 5, a and b). DNA laddering assays confirmed that $\beta_1\text{AR}$ -induced DNA fragmentation was resistant to the PKA inhibitor PKI, but suppressed by the CaMKII inhibitor KN93 (Figure 5c). Moreover, in either $\beta_2\text{AR}$ KO or WT mouse cardiac myocytes, inhibition of CaMKII by AIP or KN93 similarly prevented $\beta_1\text{AR}$ -mediated apoptosis (Figure 2). Norepinephrine (at $1 \mu\text{M}$), a physiological catecholamine, in the presence of an $\alpha_1\text{AR}$ blocker, prazosin ($1 \mu\text{M}$), similarly promoted apoptosis in WT cells, an effect that was also abolished by KN93 or AIP (data not shown). These results provide the first demonstration

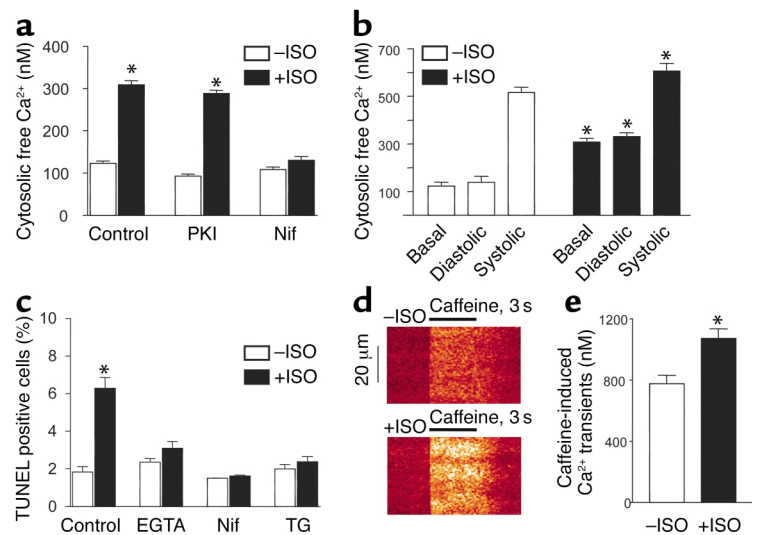


Figure 4

PKA-independent increase in intracellular Ca^{2+} is essential for the $\beta_1\text{AR}$ apoptotic effect. After $\beta_1\beta_2$ DKO myocytes were infected by Adv- $\beta_1\text{AR}$, cells were incubated with designated reagents for 1 hour, then ISO ($1 \mu\text{M}$) was added and cells were incubated for another 3–6 hours (a, b, d, and e) or 24 hours (c). (a) Prolonged $\beta_1\text{AR}$ stimulation elevated basal intracellular free Ca^{2+} in unpaced cardiac myocytes. This effect was blocked by the L-type Ca^{2+} channel antagonist nifedipine ($1 \mu\text{M}$), but not the PKA inhibitor PKI ($5 \mu\text{M}$). $*P < 0.01$ vs. ISO-untreated groups and those pretreated by nifedipine ($n = 20$ –35 cells from six hearts). (b) Intracellular Ca^{2+} transients were measured in a subset of cells electrically paced at 0.5 Hz for at least 10 minutes in the absence ($n = 29$ cells from four hearts) and presence ($n = 22$ cells from four hearts) of sustained $\beta_1\text{AR}$ stimulation by ISO. $*P < 0.05$ vs. ISO-untreated myocytes. (c) Effects of nifedipine, EGTA-AM ($1 \mu\text{M}$), or the SR ATPase inhibitor thapsigargin ($1 \mu\text{M}$) on $\beta_1\text{AR}$ -induced increase in TUNEL-positive cells. $*P < 0.01$ vs. ISO-untreated myocytes and those pretreated with EGTA-AM, nifedipine, or thapsigargin ($n = 4$ –8). (d) Representative confocal linescan images of caffeine-elicited SR Ca^{2+} release in ISO-treated ($1 \mu\text{M}$, 3 hours, bottom) and untreated cells (top). The x axis shows the time courses for caffeine treatment, and the y axis shows the spatial profiles of Ca^{2+} transients along a scan line inside the cell. (e) Average amplitude of caffeine-elicited Ca^{2+} transients in ISO-treated or untreated group. $*P < 0.01$ vs. ISO-untreated myocytes. $n = 25$ –30 cells from six hearts in each group. Nif, nifedipine; TG, thapsigargin.

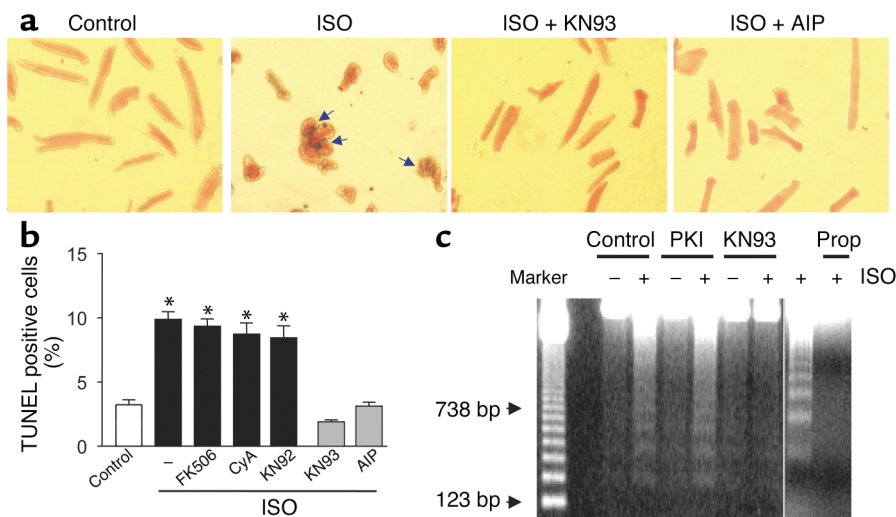


Figure 5

Role of CaMKII and calcineurin in the β_1 AR-mediated apoptotic effect. After $\beta_1\beta_2$ DKO myocytes were infected by Adv- β_1 AR, cells were pretreated with the CaMKII inhibitors AIP (10 μ M) or KN93 (0.5 μ M) or the inactive KN93 analogue KN92 (2 μ M); with the calcineurin inhibitors FK506 (10 μ M) or cyclosporin A (5 μ M); with the PKA inhibitor PKI (5 μ M); or with the Ca^{2+} channel blocker nifedipine (1 μ M), all for 1 hour (except 3 hours for AIP) prior to administration of 1 μ M ISO. Apoptosis was assessed after incubation for another 24 hours. (a) AIP or KN93 fully protected cardiomyocytes against ISO-elicited apoptosis. Arrows indicate TUNEL-positive nuclei. (b) The ISO-induced increase in TUNEL-positive cells was prevented by AIP or KN93, but not the inactive KN93 analogue KN92 or the calcineurin inhibitors FK506 or cyclosporin A. $n = 4-8$. $*P < 0.01$ versus ISO-untreated groups or those treated with KN93 or AIP. (c) ISO-induced DNA laddering in the absence (control) or presence of KN93, PKI, or the β AR antagonist propranolol. Similar results were obtained in four other experiments. CyA, cyclosporin A.

that activation of CaMKII, rather than PKA, is required for β_1 AR-induced apoptosis in the heart.

The pivotal role of CaMKII in β_1 AR-mediated apoptotic signaling was reinforced by data on the pharmacological profile of β_1 AR-induced CaMKII activation. In Adv- β_1 AR-infected DKO cells, ISO stimulation for 6 hours increased the level of autophosphorylated CaMKII, an active form of the kinase (36), without altering the abundance of the kinase protein (Figure 6a). Notably, the increase in CaMKII activity was PKI-resistant, but was abolished by KN93 and AIP (Figure 6, a-c) at the same respective concentrations that blocked the β_1 AR-mediated apoptotic effect. Since CaMKII activity was elevated prior to the manifestation of cardiomyocyte apoptosis, we conclude that CaMKII activation constitutes an early β_1 AR-elicited event to signal cell apoptosis.

Overexpression of CaMKII- δ C exaggerates β_1 AR-induced apoptosis. Based on the aforementioned results, an increase in CaMKII abundance should enhance the β_1 AR apoptotic effect in cardiac myocytes. To test this hypothesis, we expressed HA-tagged CaMKII- δ C, a predominant cardiac cytoplasmic CaMKII isoform (37), or CaMKII- δ B, a nuclear CaMKII isoform (37), in β_2 AR KO myocytes. Confocal immunocytochemical analysis confirmed that the expressed CaMKII- δ C was distributed in cytoplasm and the surface membranes, including the transverse tubules (Figure 7a, II and III),

but not in the nuclei (Figure 7a, III), whereas CaMKII- δ B was concentrated in the nuclei (Figure 7a, IV). Expression of HA-tagged CaMKII- δ C was also confirmed by Western blotting using a monoclonal antibody reacting with HA (Figure 7b). Remarkably, overexpression of CaMKII- δ C shifted the dose-response curve of ISO-induced apoptosis leftward by nearly an order of magnitude (EC_{50} , 1.1 nM and 9.0 nM for myocytes infected by Adv-CaMKII- δ C and Adv- β gal, respectively) and increased the maximal apoptotic effect by 50%, whereas it exerted no appreciable apoptotic effect in the absence of β_1 AR stimulation by ISO (Figure 7c). Again, inhibition of CaMKII by KN93 in cardiac myocytes overexpressing CaMKII- δ C abolished the β_1 AR apoptotic effect over a wide range of agonist concentrations (Figure 7c). By contrast, overexpression of

CaMKII- δ B neither enhanced nor reduced β_1 AR-induced myocyte apoptosis (data not shown), indicating that CaMKII- δ C but not CaMKII- δ B is involved in β_1 AR apoptotic signaling. The fact that increased CaMKII- δ C abundance exaggerates the β_1 AR apoptotic effect without altering basal cell apoptosis substantiates the conclusion that CaMKII mediates the β_1 AR apoptotic signal.

Discussion

β_1 AR apoptotic signaling pathway. The prevalent theory of β_1 AR signal transduction is that the cAMP/PKA pathway is solely responsible for β_1 AR-mediated cellular responses. A close inspection of studies to date, however, reveals no convincing evidence to validate that this is also the case for β_1 AR-evoked apoptotic signal in the heart. Using two genetically defined β_1 AR systems, we have provided the first documentation that sustained β_1 AR stimulation delivers a powerful cardiac apoptotic signal via a CaMKII-dependent, rather than a PKA-dependent, mechanism. This conclusion is based on several lines of evidence. First, inhibition of PKA by a specific peptide inhibitor, PKI, or an inactive cAMP analogue, Rp-cAMP, does not affect β_1 AR-induced myocyte apoptosis under conditions in which PKA-mediated protein phosphorylation is completely blocked. Second, sustained β_1 AR stimulation elicits PKA-independent augmentation of intracellular Ca^{2+} as well as SR Ca^{2+}

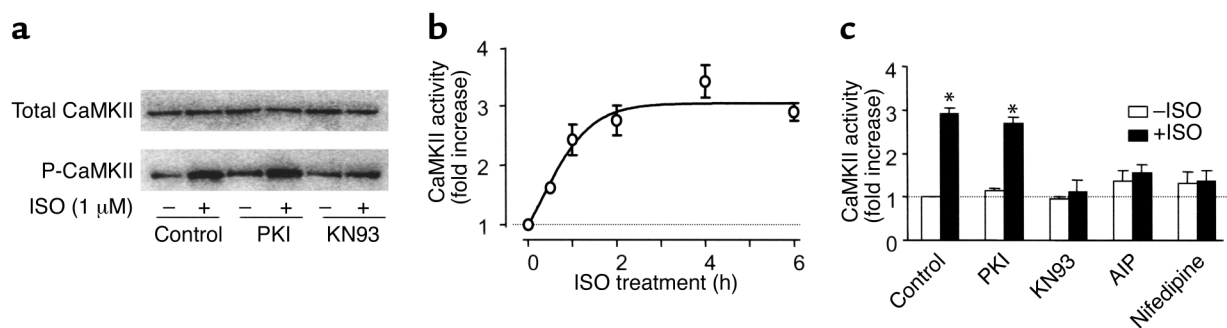


Figure 6 Temporal and pharmacological profiles of CaMKII activation in response to β_1 AR stimulation. (a) In Adv- β_1 AR-infected $\beta_1\beta_2$ DKO myocytes, β_1 AR stimulation (1 μ M ISO for 6 hours) increased CaMKII autophosphorylation. This effect was blocked by KN93 (5 μ M) but not by the PKA inhibitor PKI (5 μ M). Similar results were obtained in three other experiments. (b) Time course of ISO-induced increase in CaMKII activity assayed by 32 P incorporation into a specific peptide substrate of the kinase (see Methods; $n = 4$ for each data point). (c) Pharmacological profile of CaMKII activation ($n = 4-6$). * $P < 0.01$ vs. cells in the absence of ISO or those in the presence of KN93, AIP, or nifedipine. P-CaMKII, autophosphorylated CaMKII.

load in both resting and electrically paced myocytes, and CaMKII is activated in a time-dependent fashion. More importantly, either blocking I_{Ca} , buffering intracellular Ca^{2+} , or inhibiting CaMKII activity fully protects cardiac myocytes against β_1 AR-induced apoptosis. The essential role of CaMKII in β_1 AR apoptotic signaling is also corroborated by the fact that overexpression of a cardiac isoform of CaMKII, CaMKII- δ C, markedly enhances β_1 AR-induced apoptosis. Similarly, in cultured adult rat cardiac myocytes, β_1 AR-mediated apoptosis is blocked by nifedipine or CaMKII inhibition with KN93, but not by PKA inhibition with PKI (data not shown). Thus, we conclude that the β_1 AR-evoked apoptotic signal is delivered by a PKA-independent, CaMKII-mediated signaling pathway. Nevertheless, this should not be taken as evidence that basal CaMKII activation (such as in the beating heart) alone is sufficient to induce apoptosis. Our previous studies have shown that 2.0-Hz electrical pacing is able to augment CaMKII activation, as shown by a twofold increase in CaMKII-dependent phosphorylation of PLB at Thr¹⁷, and that the effect of pacing on PLB-Thr¹⁷ phosphorylation is synergistically enhanced to sixfold when combined with β_1 AR stimulation in freshly isolated rat cardiac myocytes (38), indicating that β_1 AR stimulation and electrical pacing exert a synergistic effect on CaMKII activation in cardiac myocytes.

Although unexpected, the present finding that the β_1 AR apoptotic effect is PKA-independent is supported by previous observations that transgenic overexpression of adenylyl cyclase and the resultant elevation of intracellular cAMP in mouse hearts are dissociated from myocyte apoptosis (14, 15), and that β_2 AR stimulation exhibits a profound antiapoptotic effect despite elevated intracellular cAMP formation in cardiac myocytes (3-5, 16). Together, the present and previous studies indicate that an increase in cAMP/PKA signaling does not necessarily cause heart cell apoptosis. This perhaps reflects distinct compartmentation of intracellular cAMP under different circumstances (2, 26, 39-42). In

contrast, the linkage of sustained β_1 AR stimulation to cardiac myocyte apoptosis by the multifunctional protein kinase, CaMKII, is in general agreement with recent findings that, in naive cell lines, numerous insulting factors including UV light, TNF- α (43), and protein phosphatase inhibitors (44) can activate CaMKII, which then contributes to the insult-induced apoptosis.

Distinct signaling modes underlie sustained versus acute β_1 AR stimulation. Given that both Ca^{2+} and cAMP serve as the second messenger, β_1 AR signal transduction bifurcates into two pathways mediated by CaMKII and PKA, respectively. Interestingly, the two pathways are called upon in tandem to fulfill distinctly different functional roles. Acute β_1 AR stimulation rapidly

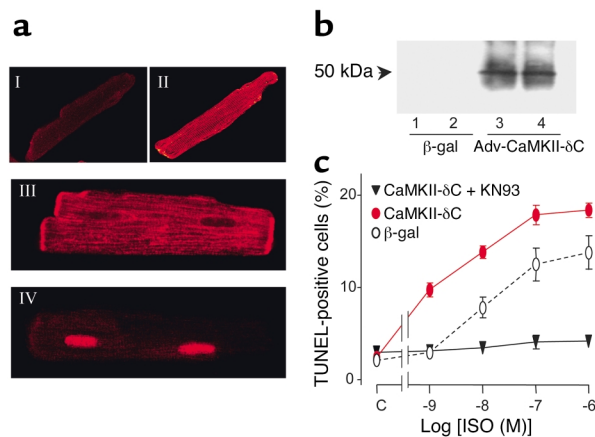


Figure 7 Overexpression of CaMKII- δ C exaggerates β_1 AR-induced myocyte apoptosis. (a) Confocal imaging of HA immunofluorescence in typical β_2 AR KO myocytes expressing either β -gal (I), or HA-tagged CaMKII- δ C (II, cell surface scan; III, cell nucleus level scan), or HA-tagged CaMKII- δ B (IV). (b) Expression of HA-tagged CaMKII- δ C assayed by Western blot with an antibody reacting with HA. (c) Dose responses of β_1 AR-induced increase in apoptotic cells in β_2 AR KO myocytes infected by Adv-CaMKII- δ C (with or without KN93) or Adv- β -gal ($n = 6$ for each data point).

activates the cAMP/PKA pathway, with the peak response within 1 minute (45), whereas prolonged β_1 AR stimulation causes desensitization of cAMP/PKA signaling within 30 minutes (1, 24). This fast cAMP/PKA response is crucial to sympathetic control over the heart rate and myocardial contraction, allowing the heart to increase its output within seconds in response to a “fight-or-flight” situation. In contrast, the newly identified CaMKII signaling is evoked gradually and appears to contribute little to acute β_1 AR contractile response, but is essential to the β_1 AR cardiac apoptotic effect.

The slow kinetics of CaMKII activation may reflect a cumulative increase in intracellular Ca^{2+} due to a small but persistent Ca^{2+} entry through L-type channels. This interpretation is consistent with the facts that CaMKII activation is blocked by the L-type channel antagonist nifedipine and that CaMKII- δC is enriched beneath cell surface membranes, thus permitting an intimate interplay between CaMKII and the L-type channel (23). Alternatively, the gradual elevation of CaMKII activity may reflect that multiple steps, e.g., upregulation of intermediate signaling components, are involved in transducing receptor signal to the kinase activation.

Regardless of the exact mechanism, the time-dependent switch of signaling modes during enduring receptor stimulation may represent a new paradigm of G protein-coupled receptor signal transduction. In this regard, it has recently been shown that β_2 AR stimulation induces a switch from cAMP/PKA signaling to a G_i -dependent MAPK signaling pathway (46), and that these two events are causally linked, i.e., activation of cAMP/PKA is a prerequisite for the receptor coupling to G_i proteins and the subsequent activation of the MAPK pathway (46). In the case for sustained β_1 AR stimulation, however, the gradual activation of CaMKII is independent of PKA signaling (Figure 6). Both examples show a time-dependent homologous regulation of G protein-coupled receptor signaling.

Regarding possible mechanisms underlying the non-PKA-dependent increase in intracellular Ca^{2+} , it has been proposed that persistent β_1 AR stimulation increases I_{Ca} via a direct coupling of $G_{\text{s}\alpha}$ to the Ca^{2+} channel (47). More recently, it has been shown that cardiac-specific overexpression of $G_{\text{s}\alpha}$ similarly augments I_{Ca} amplitude in a PKA-independent manner (48). Both lines of evidence support the idea that PKA-independent cross-talk between $G_{\text{s}\alpha}$ and the L-type Ca^{2+} channel may contribute to β_1 AR-induced increase in intracellular Ca^{2+} and subsequent activation of CaMKII.

Pathophysiological relevance of β_1 AR apoptotic signaling. Increasing evidence indicates that prolonged β_1 AR stimulation exerts a cardiotoxic effect that often outweighs the short-term gain in cardiac contractile support. Both in vivo and in vitro studies have shown that enhanced β_1 AR signaling by either selective receptor stimulation or receptor overexpression increases proapoptotic protein levels and promotes cardiac apoptosis (3–6). Since cardiac myocytes are terminally

differentiated cells, preventing the loss of irreplaceable cells is critical for the maintenance of normal cardiac function. These studies explain, at least in part, the poor prognosis of heart failure patients with tonically elevated plasma norepinephrine, an endogenous β_1 AR agonist (49), as well as the beneficial effects of β AR blockers, particularly β_1 AR blockers in chronic heart failure (50). In light of the present findings, an increase in intracellular Ca^{2+} and subsequent activation of CaMKII, rather than the long-suspected cAMP/PKA cascade, are particularly cardiotoxic in terms of cardiac myocyte apoptosis. Thus, unraveling the new pathway and the novel signaling mode of β_1 AR signal transduction hold promise for identifying key therapeutic targets and new strategies for minimizing detrimental effects of β_1 AR stimulation in the failing heart.

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